

Lactose/D-Galactose

UV-method

for the determination of lactose and D-galactose in food-stuffs and other materials

Cat. No. 10 176 303 035

Test-Combination for 32 determinations

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

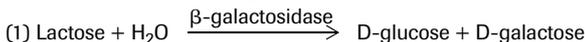
For use in *in vitro* only

Store at 2-8° C

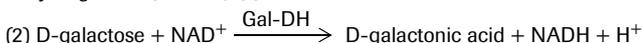
For recommendations for methods and standardized procedures see references (A 2, B 2)

Principle (Ref. A1)

Lactose is hydrolyzed to D-glucose and D-galactose at pH 6.6 in the presence of the enzyme β -galactosidase and water (1).



D-Galactose is oxidized at pH 8.6 by nicotinamide-adenine dinucleotide (NAD) to D-galactonic acid in the presence of the enzyme β -galactose dehydrogenase (Gal-DH) (2).



The amount of NADH formed in reaction (2) is stoichiometric to the amount of lactose, and D-galactose, resp. The increase in NADH is measured by means of its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 600 mg lyophilizate, consisting of: citrate buffer, pH approx. 6.6; NAD, approx. 35 mg; magnesium sulfate
2. Bottle 2 with approx. 1.7 ml β -galactosidase suspension, approx. 100 U
3. Bottle 3 with approx. 34 ml solution, consisting of: potassium diphosphate buffer, pH approx. 8.6
4. Bottle 4 with approx. 1.7 ml galactose dehydrogenase suspension, approx. 40 U
5. Bottle 5 with lactose assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Dissolve contents of bottle 1 with 70 ml redist. water.
2. Use suspension of bottle 2 undiluted.
3. Use solution of bottle 3 undiluted.
4. Use suspension of bottle 4 undiluted.

Stability of reagents

The contents of bottle 1 are stable at 2-8°C (see pack label).

Solution 1 is stable for 3 months at 2-8°C.

Bring solution 1 to 20-25°C before use.

The contents of bottles 2, 3 and 4 are stable at 2-8°C (see pack label).

Bring solution 3 to 20-25°C before use.

Procedure

Wavelength¹: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette²: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.300 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 4-200 μg lactose and D-galactose/assay³
in 0.100-0.500 ml sample volume

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.

2 If desired, disposable cuvettes may be used instead of glass cuvettes.

3 See instructions for performance of assay

4 The performance of blank D-galactose sample and D-galactose sample can be omitted if the sample does not contain free D-galactose.

Pipette into cuvettes	Blank lactose sample	Lactose sample	Blank ⁴ D-galactose sample	D-Galactose sample ⁴
solution 1* suspension 2 sample solution**	0.200 ml 0.050 ml -	0.200 ml 0.050 ml 0.100 ml	0.200 ml - -	0.200 ml - 0.100 ml
Mix* and incubate for 20 min at 20-25°C. Add:				
solution 3 redist. water	1.000 ml 2.000 ml	1.000 ml 1.900 ml	1.000 ml 2.050 ml	1.000 ml 1.950 ml
Mix***, after approx. 2 min read absorbances of the solutions (A ₁). Start reaction by addition of:				
suspension 4	0.050 ml	0.050 ml	0.050 ml	0.050 ml
Mix***, wait until reaction has stopped (approx. 15 min with 50 μg lactose/assay and approx. 30 min with 200 μg lactose/assay) and read absorbances of the solutions (A ₂). If the reaction has not stopped after 15 min, continue to read the absorbances at 5 min intervals until the absorbance increases constantly over 5 min.				

* Pipette solution 1, suspension 2 and sample solution, each onto the bottom of the cuvette, mix by swirling. When using a plastic spatula, remove it from the cuvette only directly before measuring absorbance A₁.

** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

If the absorbance A₂ increases constantly, extrapolate the absorbance to the time of the addition of suspension 4 (β -galactose dehydrogenase).

Determine the absorbance differences (A₂-A₁) for both, blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample.

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

It follows $\Delta A_{\text{D-galactose}}$ (from "D-galactose sample") and

$\Delta A_{\text{lactose + D-galactose}}$ (from "lactose sample")

The difference of these values stands for $\Delta A_{\text{lactose}}$.

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt. 4).

Calculation

According to the general equation for calculating the concentrations:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ϵ = extinction coefficient of NADH at:

$$340 \text{ nm} = 6.3 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 365 \text{ nm} = 3.4 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 334 \text{ nm} = 6.18 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

It follows for lactose (calculated as anhydrous lactose):

$$c = \frac{3.300 \times 342.3}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{\text{lactose}} = \frac{11.30}{\epsilon} \times \Delta A_{\text{lactose}} \text{ [g lactose/l sample solution]}$$

for lactose (calculated as lactose monohydrate):

$$c = \frac{3.300 \times 360.32}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{\text{lactose}} = \frac{11.89}{\epsilon} \times \Delta A_{\text{lactose}} \left[\frac{\text{g lactose}}{\text{H}_2\text{O/l sample solution}} \right]$$

for D-galactose:

$$c = \frac{3.300 \times 180.16}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{\text{galactose}} = \frac{5.945}{\epsilon} \times \Delta A_{\text{D-galactose}} \left[\frac{\text{g D-galactose}}{\text{l sample solution}} \right]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{lactose}} = \frac{c_{\text{lactose}} \left[\frac{\text{g/l sample solution}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \right]}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 \left[\frac{\text{g}}{100 \text{ g}} \right]$$

$$\text{Content}_{\text{D-galactose}} = \frac{c_{\text{D-galactose}} \left[\frac{\text{g/l sample solution}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \right]}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 \left[\frac{\text{g}}{100 \text{ g}} \right]$$

1. Instructions for performance of assay

The amount of lactose and D-galactose present in the assay has to be between 10 µg and 200 µg (measurement at 365 nm) or 4 µg and 100 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a lactose and D-galactose concentration between 0.3 and 2 g/l or 0.2 and 1 g/l, respectively.

Dilution table

Estimated amount of lactose and D-galactose per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 1.0 g	< 2.0 g	-	1
1.0-10.0 g	2.0-20.0 g	1 + 9	10
10.0-100 g	20.0-200 g	1 + 99	100
> 100 g	> 200 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 0.500 ml (neutralize sample previously, if necessary). The volume of water added must then be reduced so as to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

- Many foodstuffs do not contain free D-galactose. The determination of D-galactose can therefore be omitted.
- If only D-galactose is to be determined (or L-arabinose, see pt. 11), the assay procedure may be continued without incubation after addition of the sample solution.
- The calculation of the results can be done on the basis of anhydrous lactose (molecular weight 342.3), which can be recommended, and on the basis of lactose monohydrate (molecular weight 360.32). When comparing data, one has to make sure that the same molecular weight has been used for the calculation (figures for lactose monohydrate are 5% higher than those for anhydrous lactose).

3. Specificity

β -Galactosidase hydrolyzes β -galactosidic bonds e.g. in lactose and with reduced speed in lactulose with the uptake of water.

Apart from D-galactose, Gal-DH also oxidizes L-arabinose. Small amounts of free D-galactose and L-arabinose only occur in foodstuffs, if they have been released from their natural glycosidic linkage (e.g. of swelling materials) due to chemical or enzymatic influences.

In the analysis of commercial lactose monohydrate (molecular weight 360.32) and of D-galactose (molecular weight 180.16) results of < 100% have to be expected because the materials absorb moisture.

4. Sensitivity and detection limit

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume $v = 0.500$ ml and measurement at 340 nm of a lactose concentration of 2 mg/l,

resp. 1 mg D-galactose/l sample solution (if $v = 0.100$ ml, this corresponds to 10 mg lactose/l, resp. 5 mg D-galactose/l sample solution).

The detection limit of 7 mg lactose/l, resp. 4 mg D-galactose/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume $v = 0.500$ ml.

5. Linearity

Linearity of the determination exists from approx. 4 µg lactose + D-galactose/assay (7 mg lactose + D-galactose/l sample solution; sample volume $v = 0.500$ ml) to 200 µg lactose + D-galactose/assay (2 g lactose + D-galactose/l sample solution; sample volume $v = 0.100$ ml).

6. Precision

In a double determination of D-galactose using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of $v = 0.100$ ml and measurement at 340 nm, this corresponds to a D-galactose concentration of approx. 5-10 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/ 100 ml = 10 g/l, a difference of 0.05-0.1 g/100 g can be expected.)

In a double determination of lactose using one sample solution, a difference of 0.010 to 0.015 absorbance units may occur in the presence of D-galactose in the sample. With a sample volume of $v = 0.100$ ml and measurement at 340 nm, this corresponds to a lactose concentration of approx. 15-25 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.15-0.25 g/100 g can be expected.)

The following data for the determination of lactose and D-galactose have been published in the literature:

Lactose in chocolate:

$$x = 6.01 \text{ g/100 g} \quad s = 0.04 \text{ g/100 g} \text{ CV} = 0.7 \% \quad (\text{Ref. A 1.1})$$

$$25 \text{ } \mu\text{g D-galactose/assay CV} = 0.95 \%$$

$$85 \text{ } \mu\text{g lactose/assay CV} = 1.13 \%$$

$$50 \text{ } \mu\text{g D-galactose/assay CV} = 0.64 \%$$

$$170 \text{ } \mu\text{g lactose/assay CV} = 0.78 \% \quad (\text{Ref. A 1.2})$$

Milk and milk products:

$$\text{Lactose: } r = 0.05 \times (\text{content}_{\text{lactose}} \text{ in g/100 g}) \text{ g/100 g}$$

$$R = 0.06 \times (\text{content}_{\text{lactose}} \text{ in g/100 g}) \text{ g/100 g}$$

$$\text{D-Galactose: } r = 0.10 \times (\text{content}_{\text{D-Galactose}} \text{ in g/100 g}) \text{ g/100 g}$$

$$R = 0.12 \times (\text{content}_{\text{D-Galactose}} \text{ in g/100 g}) \text{ g/100 g}$$

$$\text{Lactose in chocolate: } r = 0.188 \text{ g/100 g} \quad s_{(r)} = \pm 0.066 \text{ g/100 g}$$

$$R = 0.434 \text{ g/100 g} \quad s_{(R)} = \pm 0.153 \text{ g/100 g} \quad (\text{Ref. A 2.5})$$

For further data see references.

7. Recognizing interference during the assay procedure

- If the conversion of D-galactose has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.
- On completion of the reaction, the determination can be restarted by adding D-galactose (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

The reaction cannot be restarted with lactose as, subsequent to altering the reaction conditions from pH 6.6 to pH 8.6 ("change of the buffer"), lactose is no longer cleaved.

- Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

The use of "single" and "double" sample volumes in double determinations is the simplest method of carrying out a control assay in the determination of lactose.

- Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

7.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

8. Reagent hazard

The reagents used in the determination of lactose and D-galactose are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

9. General information on sample preparation

In carrying out the assay:

Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 0.500 ml; Filter **turbid solutions**;

Degas **samples containing carbon dioxide** (e.g. by filtration);

Treat **“strongly colored” samples** that are used undiluted or with a higher sample volume with polyvinylpyrrolidone (PVPP) or with polyamide, e.g. 1 g/100 ml;

Crush or homogenize **solid or semi-solid samples**, extract with hot water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;

Deproteinize **samples containing protein** with perchloric acid or with trichloroacetic acid; alternatively clarify with Carrez reagents;

Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez-solutions after the extraction with hot water;

Break **emulsions** with trichloroacetic acid or clarify with Carrez-solutions.

Carrez clarification:

Pipette the liquid sample into a 100 ml volumetric flask which contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g $K_4[Fe(CN)_6] \times 3 H_2O/100$ ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 720 g $ZnSO_4 \times 7 H_2O/100$ ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

10. Application examples

Determination of lactose in drinking milk, condensed milk and sour milk

Accurately weigh approx. 2 g of milk into a 100 ml volumetric flask. Dilute with approx. 20 ml water and add 1.0 ml trichloroacetic acid (3 M) for protein precipitation. After 10 min incubation neutralize with NaOH (1 M), fill up to 100 ml with water, filter and use the clear, possibly slightly opalescent solution for the assay.

In the case of sour milk, there is no need for deproteinization.

When lactose is determined in drinking milk or condensed milk, clarification can also be carried out with Carrez reagents (see “determination of lactose in yogurt”). Amount weighed: approx. 2 g.

Determination of lactose in hard cheese or chocolate

Grate cheese crumbs or chocolate, accurately weigh approx. 2 g into a 100 ml volumetric flask, add approx. 70 ml water and incubate for 15 min at approx. 70°C. Shake from time to time. After cooling to 20-25°C, fill up to the mark with water and mix. To separate the fat, place in a refrigerator for approx. 20 min and filter. Discard the first few ml. Use the clear, possibly slightly opalescent solution for the assay.

Determination of lactose in processed cheese

Accurately weigh approx. 3 g of homogeneous sample into a 100 ml volumetric flask, add approx. 70 ml water and incubate for 15 min at approx. 70°C while continuously stirring (magnetic stirrer). After cooling to 20-25°C, add 2 ml trichloroacetic acid (3 M) and mix. Neutralize with approx. 6 ml NaOH (1 M), fill up to the mark with water.

For the separation of fat, place in a refrigerator for approx. 20 min, filter or centrifuge. Use the clear, possibly slightly opalescent solution for the assay.

Alternatively the procedure “determination of lactose in yogurt” can also be used. Amount weighed: approx. 3 g.

Determination of lactose in instant baby food, restorative food or ice cream

Accurately weigh approx. 1 g of sample into a 100 ml volumetric flask, add approx. 60 ml water and incubate for 15 min at approx. 70°C, shake from time to time. After cooling to 20-25°C, add 10 ml perchloric acid (3 M) and mix. After 10 min neutralize with KOH (3 M) to pH 7, fill up to the mark with water and place in a refrigerator for 20 min to precipitate the $KClO_4$ formed, filter. Use the clear, possibly slightly turbid solution for the assay, diluted, if necessary.

Determination of lactose in yogurt, cream powder, milk powder and whey powder

Accurately weigh approx. 1 g of sample into a 100 ml volumetric flask, add approx. 60 ml water and incubate for 15 min at approx. 70°C; shake from time to time. For clarification, add 5 ml of Carrez-I-solution (3.60 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \times 3 H_2O/100$ ml), 5 ml of Carrez-II-solution (720 g of zinc sulfate, $ZnSO_4 \times 7 H_2O/100$ ml) and 10 ml of NaOH (0.1 M); mix after each addition, adjust to 20-25°C and fill up to the mark with water, filter. Use the clear, possibly slightly turbid solution for the assay, diluted, if necessary.

Determination of lactose in meat sausage and meat loaf

Accurately weigh approx. 5 g of cut or grated sample (mixer) into a 100 ml volumetric flask, add about 70 ml water and incubate for 15 min at approx. 70°C. Allow to cool to 20-25°C and fill up to the mark with water. For the separation of fat, place in a refrigerator for 20 min and filter. Use the clear, possibly slightly turbid solution for the assay.

Determination of lactose in samples containing protein

Add perchloric acid (1 M) to protein-containing sample solutions in a ratio of 1:3 (1+2), mix, centrifuge, and neutralize an aliquot portion of the supernatant solution with KOH (2 M). Fill up to a certain volume in the volumetric flask, place in a refrigerator for 20 min to precipitate the $KClO_4$ and filter. Use the clear solution for the assay, diluted, if necessary.

11. Determination of L-arabinose

In the absence of D-galactose, the Gal-DH reaction can be used to determine L-arabinose. Then the reaction time rises to 30 min (after addition of suspension 4).

12. Further applications

The method may also be used in the examination of pharmaceuticals (Ref. A 4.1) and in research when analyzing biological samples. For details of sampling, treatment and stability of the sample see Ref. A 1.1.

12.1. Determination of lactose in the urine of cows (Ref. A 4.2)

Dilute urine according to the dilution table and use it for the assay (dilution factor = F)

Important note: Incubate the mixture of solution 1, suspension 2 and the sample solution at 20-25°C for 30 min.

Calculation:

$$c = \frac{11.30 \times \Delta A \times F}{\epsilon} \text{ [g lactose/l sample]}$$

$$c = \frac{33.00 \times \Delta A \times F}{\epsilon} \text{ [mmol lactose/l sample]}$$

Wavelength	Hg 365 nm	340 nm	Hg 334 nm
c [g/l]	$3.324 \times \Delta A \times F$	$1.794 \times \Delta A \times F$	$1.828 \times \Delta A \times F$
c [mM]	$9.706 \times \Delta A \times F$	$5.238 \times \Delta A \times F$	$5.340 \times \Delta A \times F$

12.2 Determination of lactose in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) in a water-bath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with perchloric acid or with Carrez-solutions. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

A. References for the determination of lactose and D-galactose

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Lactose assay control solution (Bottle 5)

Concentration*: see bottle label

Lactose assay control solution is a stabilized aqueous solution of lactose. It serves as an assay control solution for the enzymatic determination of lactose in foodstuffs and other materials.

Application:

1. Addition of lactose assay control solution to the assay mixture:

Instead of the sample solution the assay control solution is used for the assay.

2. Restart of the reaction, quantitatively:

A restart of the reaction with assay control solution after completion of the reaction cannot be performed as under the assay conditions (potassium diphosphate buffer, pH 8.6) lactose is not cleaved. If necessary, restart with 0.050 ml of a D-galactose solution (0.5 g/l)

3. Internal standard:

The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank lactose sample	Lactose sample	Lactose standard	Lactose sample + standard
solution 1	0.200 ml	0.200 ml	0.200 ml	0.200 ml
suspension 2	0.050 ml	0.050 ml	0.050 ml	0.050 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml

Mix. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100 [\%]$$

*Stated as anhydrous lactose



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